Biochemical activities of *Arabidopsis* RNA-dependent RNA polymerase 6

Julien Curaba and Xuemei Chen

SUPPLEMENTAL DATA

FIGURE S1. Purification of HA-RDR6 and HA-RDR6m from plants. Western blots were performed with anti-HA antibodies. (A) *N. benthamiana* leaves infiltrated with *Agrobacteria* harboring *35S::HA-RDR6* or *35S::HA-RDR6m* constructs were harvested 2 days after infiltration. Immunoprecipitation (IP) was performed with 1 ml of total protein extract and 50 μl of anti-HA agarose beads. 20 μl of the total extract (lanes 1 and 3) and 1 μl of the agarose beads after IP (lanes 2 and 4) were loaded on a 8% polyacrylamide-SDS gel. (B) A comparison of protein quantities. HA-RDR6 extracted from *N. benthamiana* leaves (lane 5) was compared to HA-RDR6 extracted from an *Arabidopsis* transgenic line *35S::HA-RDR6 rdr6-11* (lane 6). 1 ul of beads was loaded for each, but the IP of HA-RDR6 from *Arabidopsis* was performed with 10 times the amount of plant tissue and 50 μl of anti-HA beads. (C) A stained protein gel to illustrate the purity of the RDR6 preparation. 10 μl of HA-RDR6 beads (lane 2), 20 ng BSA (lane 3), and 40 ng BSA (lane 4), were loaded onto an 8% polyacrylamide-SDS gel. The gel was stained with SYPRO Ruby (Bio-Rad), which is more sensitive than Coomassie Blue. The RDR6 and BSA bands were indicated by an "*". Note that 1 μl of the HA-RDR6 beads was used in the activity assays.

FIGURE S2. Sequences of the luciferase/nopaline synthase (NOS) chimeric gene fragment and the primers used to generate RDR6 templates. (A) Sequence of the 3' end of the luciferase cDNA (capital letters, no underline) fused to the NOS 3' UTR (lower case letters). A miR173* sequence (capital letters with underline) was inserted in the middle to allow testing for priming activity with miR173*. This chimeric DNA was used to generate various RDR6 templates. (B) Sequences of primers used to generate PCR products of different sizes from the luciferase/NOS chimeric DNA. These PCR products were used as templates for *in vitro* transcription reactions to generate ssRNAs, which served as RDR6 templates. The pT7-F1/R1 combination was used to generate the 640F template, as were pT7-F1/R3 for 387F, pT7-F2/R1 for 435F, F2/pT7-R1 for 435R, pT7-F2/R3 for 185F, F2/pT7-R3 for 185R, F2/pT7-R2 for 246R, T40-F3/pT7-R2 for 206R-A^{40}, and T40-F3/pT7-R1 for 395R-A^{40}.

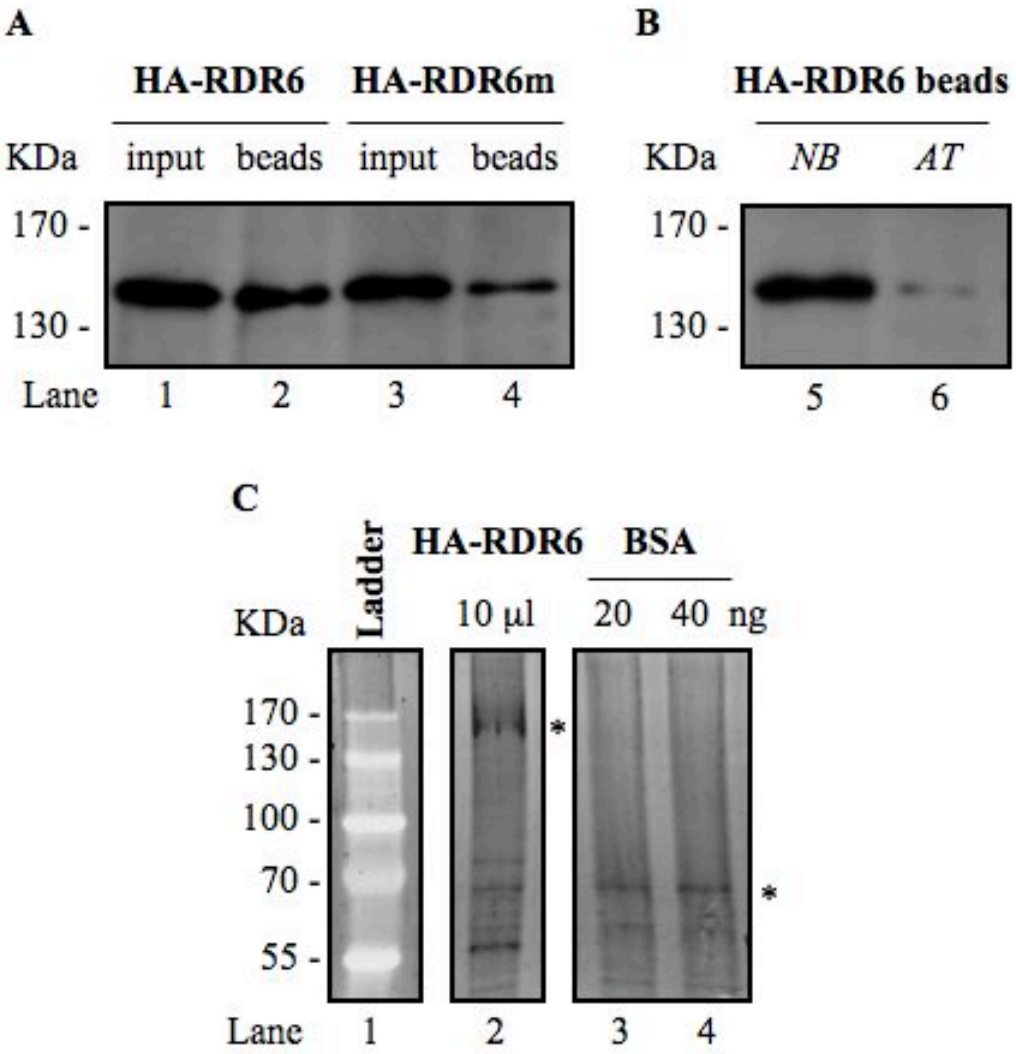
FIGURE S3. RDR6 nucleotidyl transferase activity with each individual NTP. Reactions were performed with HA-RDR6m (lane 1) and HA-RDR6 (lanes 2-5) in the presence of the 185F RNA template and one of the four [α-^{32}P] NTPs, in the absence of cold NTPs.

FIGURE S4. RDR6 activity on RNA templates of various sizes and with or without polyA tails. All enzymatic assays were performed with HA-RDR6 in the presence of [α-^{32}P]UTP and all four cold NTPs. (A) RDR6 activity was assayed on ssRNA templates that varied in sequence and in size. Each template was tested at 5 pmol, except for 387F (lane 3) at 8 pmol. (B) Assays were performed with the 185F RNA with a polyA tail

(185F-A^n; the polyA tail was introduced by yeast poly(A) polymerase) and a similarly sized 640F RNA. The reactions were performed with 4 pmol (lanes 1-2) and 8 pmol (lanes 3-4) of the templates. (C) RDR6 assays were performed on 10 pmol of ssRNA templates 395-A^{40} with (lane 1) or without (lane 2) a 5' CAP.

FIGURE S5. Effects of Mg^{2+} and Mn^{2+} on RDR6 activity. RDR assays were performed with HA-RDR6, the 246R template, [α-^{32}P]UTP, all four cold NTPs, and various quantities of divalent cations Mg^{2+} and Mn^{2+}. The final concentration of each cation in μM was indicated by the numbers above the gel images.

FIGURE S6. Treatments to generate and verify 5' end modifications of the 246R RNA template. In order to change the 5' end structure of the 246R template and to insure the strict presence of each types of RNAs (indicated under the gel image), we performed in the order, all or some of the following 4 treatments as indicated by the numbers above the gel image, in four different experiments, starting with 10 μg RNA purified from gel after *in vitro* transcription. (1) Capping reaction was performed using the ScriptCap™ m^7G Capping System (Epicentre) to convert the 5' triphosphate into a 7-methylguanosine cap. (2) Calf intestine alkaline phosphatase treatment was performed to remove the triphosphate from RNAs that failed to acquire a cap in (1). This treatment would leave a 5' OH if the RNA had a 5' triphosphate but a 5' capped RNA would be resistant to this treatment. (3) Polynucleotide kinase treatments were performed to add a monophosphate to the RNAs having a 5' OH from reaction (2). (4) Digestion was performed with the TERMINATOR enzyme (Epicentre), which specifically degraded RNAs with a 5' monophosphate. Each treatment was followed by phenol/chloroform extraction and ethanol precipitation. 1/10 of the RNA at the end of each treatment was loaded in each lane. In the last lane, in the absence of the capping reaction, 100% of the RNAs had a 5' monophosphate after reaction (3) and were completely degraded by the TERMINATOR enzyme.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HA-RDR6
HA-RDR6m
KDa
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beads
input
beads
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130 -
Lane
1
2
3
4
B
HA-RDR6 beads
KDa
NB
AT
170 -
130 -
5
6
C
Ladder
HA-RDR6
BSA
KDa
10 µl
20
40
ng
170 -
130 -
100 -
70 -
55 -
*
*
Lane
1
2
3
4

Figure S1

A

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1   - CACTTCTTCATCGTTGACCGCCTGAAGTCTCTGATTAAGTACAAAGGCTA
51  - TCAGGTGGCTCCCGCTGAATTGGAATCCATCTTGCTCCAACACCCCAACA
101- TCTTCGACGCAGGTGTCGCAGGTCTTCCCGACGATGACGCCGGTGAACTT
151- CCCGCCGCCGTTGTTGTTTTGGAGCACGGAAAGACGATGACGGAAAAAGA
201- GATCGTGGATTACGTCGCCAGTCAAGTAACAACCGCGAAAAAGTTGCGCG
251- GAGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTACCGGAAAACTC
301- GACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAA
351- GATCGCCGTGTAAtctagaGATTCTCTGTGTAAGCGAAAGtctagagtcc
401- Gcaaaaatcaccagtctctctctacaaatctatctctctctatttttctc
451- Cagaataatgtgtgagtagttcccagataagggaattagggttcttatag
501- Ggtttcgctcatgtgttgagcatataagaaacccttagtatgtatttgta
551- Tttgtaaaatacttctatcaataaaatttctaattcctaaaaccaaaatc
601- cagtgacctgcagcccgggggatccactagagcggccg
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B

Primers	Sequences (5'-3')
F1	CACTTCTTCATCGTTGACCGCC
pT7-F1	**taatacgactcactataggg**CACTTCTTCATCGTTGACCGCC
F2	CGTGGATTACGTCGCCAGTC
pT7-F2	**taatacgactcactataggg**CGTGGATTACGTCGCCAGTC
T40-F3	**tttttttttttttttttttttttttttttttttttttttt**GTTGCGCGGAGGAGTTGTGTTTG
R1	GGCCGCTCTAGTGGATCCCCC
pT7-R1	**taatacgactcactataggg**CCGCTCTAGTGGATCCCCC
R2	AGAAAAATAGAGAGAGATAG
pT7-R2	**taatacgactcactataggg**AGAAAAATAGAGAGAGATAG
R3	TTCGCTTACACAGAGAATCTC
pT7-R3	**taatacgactcactataggg**TTCGCTTACACAGAGAATCTC

Figure S2

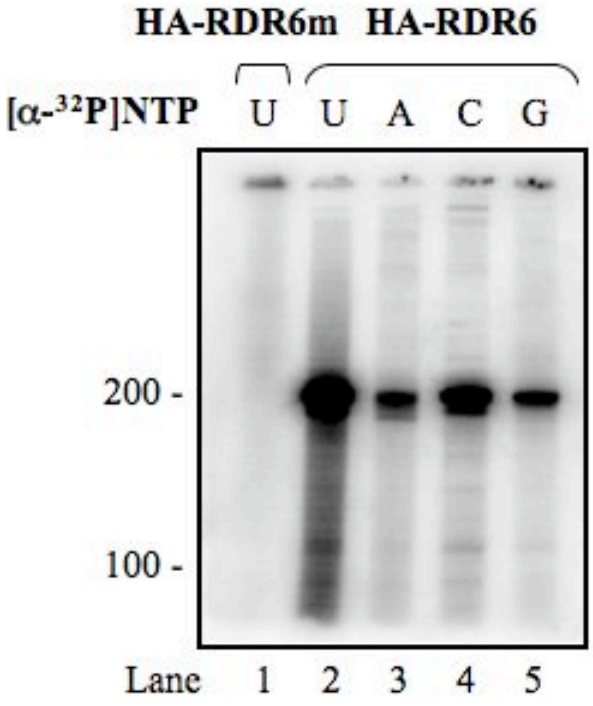
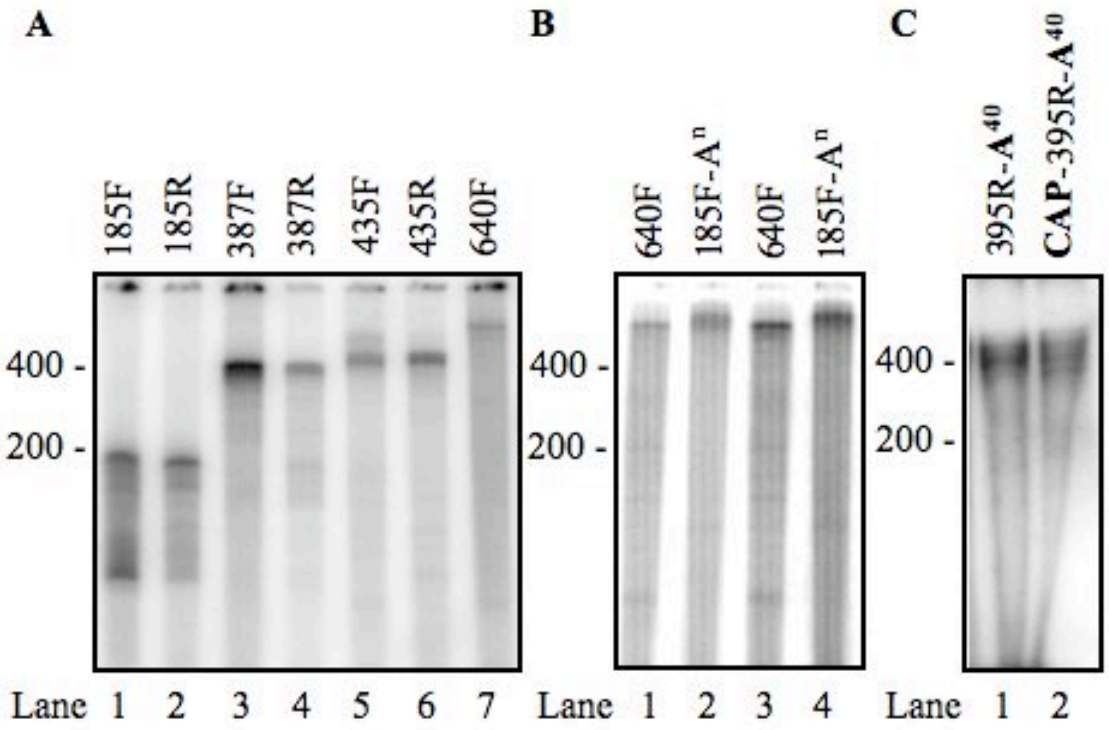


Figure S3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185F
185R
387F
387R
435F
435R
640F
400 -
200 -
Lane 1 2 3 4 5 6 7
B
640F
185F-An
640F
185F-An
400 -
200 -
Lane 1 2 3 4
C
395R-A40
CAP-395R-A40
400 -
200 -
Lane 1 2

Figure S4

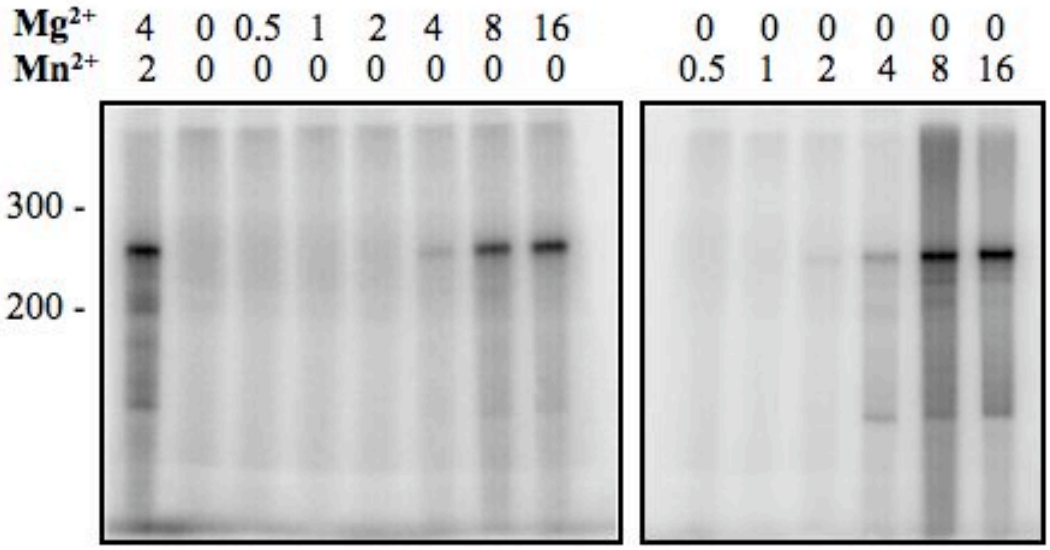

Figure S5

1-Capping reaction
2-CIP treatment
3-PNK treatment
4-Terminator digestion

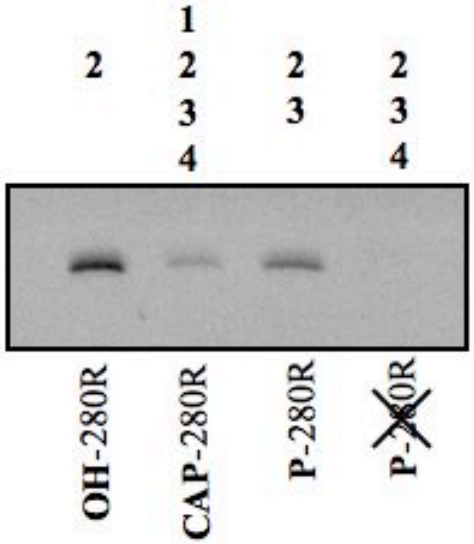

Figure S6